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Dissection of a complex seed phenotype: Novel insights of *FUSCA3* regulated developmental processes

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Dedicated to the 65th birthday of Prof. Dr. Ulrich Wobus

Abstract

A T-DNA insertion mutant of *FUSCA3* (*fus3-T*) in *Arabidopsis thaliana* exhibits several of the expected deleterious effects on seed development, but not the formation of brown seeds, a colouration which results from the accumulation of large amounts of anthocyanin. A detailed phenotypic comparison between *fus3-T* and a known splice point mutant (*fus3-3*) revealed that the seeds from both mutants do not enter dormancy and can be rescued at an immature stage. Without rescue, mature *fus3-3* seeds are non-viable, whereas those of *fus3-T* suffer only a slight loss in their germinability. A series of comparisons between the two mutants uncovered differences with respect to conditional lethality, in histological and sub-cellular features, and in the relative amounts of various storage compounds and metabolites present, leading to a further dissection of developmental processes in seeds and a partial reinterpretation of the complex seed phenotype. FUS3 function is now known to be restricted to the acquisition of embryo-dependent seed dormancy, the determination of cotyledonary cell identity, and the synthesis and accumulation of storage compounds. Based on DNA binding studies, a model is presented which can explain the differences between the mutant alleles. The *fus3-T* lesion is responsible for loss of function only, while the *fus3-3* mutation induces various pleiotropic effects conditioned by a truncation gene product causing severe mis-differentiation.

Keywords: FUSCA3; Seed development; Transcriptional regulation; EMS mutation; T-DNA insertion

Introduction

Lethal seed phenotypes of *Arabidopsis thaliana* (*At*) emerged some decades ago *via* chemically induced mutagenesis (Müller, 1963). One of the most prominent of these is associated with the accumulation of anthocyanin in the maturing and mature seed. In reference to their dark seed colouration, these mutants were named "*fusca*", the ancient Greek word for brown (Müller, 1963; Müller and Heidecker, 1968). Most *fusca* mutants suffer from limited cell expansion and seedling lethality (Misera et al., 1994). The *fus3* mutant is conditionally lethal at the late seed maturation stage, so requires rescue at an immature developmental stage. It has proven especially useful as a tool to understand the heterochronic regulation of gene expression during seed development (Müller and Heidecker, 1968; Bäumlein et al., 1994; Keith et al., 1994; Meinke et al., 1994). *FUS3* encodes a member of the B3 transcription factor family (Luerssen et al., 1998), which also includes maize *Vp1* (McCarty et al., 1991) and the *At* genes *ABI3* (Giraudat et al., 1992) and *LEC2* (Meinke et al., 1994; Stone et al., 2001). Together with the distinct CCAAT-binding *HAP3*-like *LEC1* (Meinke, 1992; Meinke et al., 1994), these interacting transcription factors act as key regulators for the accumulation of storage proteins and lipids in the seed, and for the acquisition

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of dormancy and desiccation tolerance (Koornneef et al., 1989; Finkelstein and Somerville, 1990; Meinke, 1992; Bäumlein et al., 1994; Keith et al., 1994; Meinke et al., 1994; Parcy et al., 1994; West et al., 1994; Koornneef and Karssen, 1994; Parcy et al., 1997; Raz et al., 2001; Kroj et al., 2003; Brocard-Gifford et al., 2003; Santos Mendoza et al., 2005; Kagaya et al., 2005a,b; Kotak et al., 2007; Wang et al., 2007). *FUS3* interacts with the RY-*cis*-motif CATGCA, which is present ca. 100 bp upstream of the transcription start site of many seed specific gene promoters (Bäumlein et al., 1986, 1992, 1994; Dickinson et al., 1988; Reidt et al., 2000, 2001; Mönke et al., 2004). In addition, it represents a nexus of hormone-controlled processes during plant embryogenesis (Gazzarini et al., 2004), and is thought to be required in the protoderm to restrict the domain of expression of *TTG1* (Tsuchiya et al., 2004). In particular, FUS3 has been shown to interact with RY to repress the gibberellin biosynthesis gene *AtGA3ox2* (Curaba et al., 2004). Together with *LEC1* and *LEC2*, the expression of *FUS3* is negatively regulated by the CHD3-chromatin remodelling factor PICKLE, a regulator of embryonic identity (Rider et al., 2003).

Several hypotheses for the function of FUS3 have been based on an analysis of mutant alleles arising out of chemical mutagenesis, and selected by seed phenotype. In the framework of the REGIA project (Paz-Arez et al., 2002), the phenotypic selection step has been avoided, by selecting for a T-DNA insertion in the coding region of *FUS3*. The insertion mutant identified shows both similarities and conspicuous differences with pre-existing chemically-induced mutants. We describe here a comparative analysis which distinguishes between total loss of function and the presence of a truncation product. This difference has presented an opportunity to better understand the complex *fus3* seed phenotype and to further dissect developmental processes essential for plant embryogenesis and seed development.

Materials and methods

Mutant analysis

The chemically induced mutant alleles *fus3-1* in the Di-G and the *fus3-3* allele in the Col-0 backgrounds have been described by Luerssen et al. (1998). The Col-0 alleles *fus3-10* and *fus3-11* were isolated by Raz et al. (2001). The T-DNA-insertion mutant *fus3-T* was isolated in a PCR-based screen of the AKF α-population (Sussman et al., 2000) using the primer combinations FUS3-KOa (5′ tgatgaaaatgtggaaaccaatgcctcta)/JL202 (5′cattttataataacgctgcggacatctac) and FUS3-KOb (5′ctcaacggagcccaaaccatcgaatgttc)/XR2 (5′tgggaaaacctggcgttaccc-caacttaat). The wild type allele is amplified by FUS3-KOa/FUS3-KOb. The *fus3-T* mutant has a single T-DNA insertion within the third exon of *FUS3* at position 673 (according to the MIPS unspliced CDS annotation). Re-sequencing of the insertion locus reveals that there are no extensive rearrangements at the insertion site besides a sequence of 27 bp of unknown origin right in front of the T-DNA. A precise transition from the T-DNA to the *FUS3* gene sequence was found at the 3′ end of the T-DNA insertion. The wild type accessions WS-2 and Col-0 were used as controls.

RNA isolation and RT PCR

RNA of various tissues was isolated with the RNeasy kit (Qiagen), and cDNA was amplified with the OneStep RT-PCR Kit (Qiagen). To screen for remnant transcripts in the mutants the following primers were used: FUS3-Aa (5′atggttgatgaaaatgtggaaacca 3′), FUS3-KOb (5′ctcaacggagcccaaaccatc-gaatgttc 3′), FUS3-Tr (5′gacggttttcacgtttggaccttca 3′), FUS3-Tl (5′tgaaggtc-caaacgtgaaaaccgtc 3′). The primer positions within the gene sequence are given in Fig. S1. Primer FUS3-3Ab (5′actatataagctatgctttgttaca) was used to amplify the *fus3-3* remnant transcript, corresponding to the HL1fus3-A cDNA of *fus3-1* (Luerssen et al., 1998). RT-PCR reactions were performed according to the manufacturer's instructions, with an annealing temperature of 58 °C and a cycle number of 25. To amplify SGT and SCT transcripts, the following primers were used: SGT-fw (5′aattgctagctctgtgacgtggcagaagat), SGT-rev (5′aattggatccactttaacgacatcataagc), SCT-fw (5′aaattgttccag-gagctgtgca), SCT-rev (5′atgagtggtgtgaggcccttct), Ubi-fw (5′acttcacttggtgc-caggc) and Ubi-rev (5′ccttgacgttgtcaatggtg).

Seed rescue and analysis of precocious germination

Seeds were collected from siliques at the early cotyledon stage (around 11 days after fertilisation), and plated on MS plates (Murashige and Skoog, 1962) maintained at 19 °C and 18 h light. Subsequently, plantlets were transferred to soil. For the analysis of precocious germination, at least 20 plates, each containing seeds from one silique, were assembled. The number of germinated seeds (according to the definition of Bewley and Black, 1994) was counted daily, and accumulated over 32d. This analysis was repeated at least three times (equivalent to at least 5000 seeds per genotype).

Germination of mature seeds

Seed was maintained post harvest at 37 °C, and was sampled for its germinability every other day. For the germination test, seed was surface sterilised and incubated on MS plates. Germination rates were determined after 10 days.

Immune histochemistry

Plant material (ca. 5 mm long parts of siliques containing seeds in mid to late cotyledon stage) was fixed in 2% paraformaldehyde, 0.2% glutardialdehyde and 0.01% Triton ×100 in 0.1 M cacodylic acid buffer at pH 7.4 for 3 h. After three washes of 10 min in buffer only, the samples were dehydrated through an ethanol series (10%, 30%, 50%, 70%, 90%, 96%, 100%), embedded in Unicryl resin (Polysciences, Warrington, USA) and cured at 55 °C for 3 days. Sections of thickness 1–3 μm were prepared with a RMC ultramicrotome MT-7000 (RMC, Tucson, USA) and dried onto poly L-lysine coated microscope slides (ERIE Scientific, Portsmouth, USA). Slides were stored at 4 °C until required. The material was blocked with 5% BSA, 0.1% Triton ×100, 0.175 M NaCl in Tris pH 7.4. Primary antibodies against cruciferin and napin were used as described elsewhere (Tiedemann et al., 2000). For secondary labelling, Alexa488-antiRabbit antibodies (Invitrogen/Molecular Probes, Karlsruhe, Germany) were used according to the manufacturer's instructions. The sections were counter-stained with DAPI in 0.1 M cacodylic acid buffer pH 7.4. Images were captured with a Zeiss Axioplan2 mot (Zeiss, Jena, Germany) microscope in Apotome mode, equipped with two digital cameras (AxiocamHR, AxiocamMR monochrome, both Zeiss, Jena, Germany). Multi-fluorescence images were analysed using the Axiovision software package (Zeiss, Jena, Germany). Stereo microscopic images were captured with a StereoLumarV12 stereomicroscope equipped with an AxiocamMR (colour).

Scanning electron microscopy

Mature dry seeds were attached onto carbon coated aluminium sample blocks and gold coated in an Edwards S150B sputter coater (Edwards High Vacuum Inc., Crawley, West Sussex, UK). Probes were examined under a Hitachi S4100 SEM (Hisco Europe, Ratingen, Germany) at 10 kV. Digital recordings were saved as TIFF files. Young seedlings of fus3-3 and fus3-T mutants and corresponding wild types were fixed in 2% glutaraldehyde in 50 mM phosphate buffer pH 7.0 for 2 h. After washing and dehydration through an ethanol series, samples were dried in a Bal-Tec critical point drier (Bal-Tec AG, Balzers, Switzerland), followed by gold coating in an Edwards S150B

sputter coater. Specimens were examined in a Hitachi S4100 SEM at 5 kV. Digital recordings were saved as TIFF files. Mature dry *At* seeds were split transversally with a razor blade to allow even infiltration of reagents. Seeds were chemically fixed in 2% glutaraldehyde and 2% formaldehyde in cacodylate buffer (50 mM, pH 7.0) for 2 h. After three 15 min washes with the same buffer, the probes were dehydrated through an ethanol series and embedded in Spurr's low viscosity resin. Semi-thin 1 μm sections were cut with a Reichert-Jung Ultracut S (Leica, Vienna, Austria) and stained with crystal violet. Digital recordings were made on a Zeiss Axiovert equipped with an Axiocam (Carl Zeiss, Jena, Germany). For transmission electron microscopy, ultra-thin (80 nm) sections were taken up on 50 mesh hexagonal copper grids, stained with 4% uranyl acetate and examined in a Zeiss 902 electron microscope at 80 kV.

Western blot analysis of seed proteins

Protein was extracted from equal numbers of mature seeds in 50 mM Tris–HCl buffer pH7.6 supplemented with 150 mM NaCl, 5 mM EDTA, 0.1% SDS and 0.1% β-mercaptoethanol. Protein extracts were centrifuged at 1000×*g* for 10 min. The protein extract was separated by 12.5% PAGE (Laemmli, 1970), and electrophoretically transferred to nitrocellulose membranes (0.4 μm, BA85, Schleicher and Schuell) as described previously (Borisjuk et al., 1998). After transfer, the membranes were washed in TBS and blocked for 1 h in 3% BSA. The washing step was repeated and the membranes were incubated for 1 h at room temperature in a solution of the primary antibody in TBS supplemented with 0.5% BSA and 0.05% Tween20. The membranes were washed three times for 5 min in TBS supplemented with 0.5% Triton X-100, and once without detergent. Membranes were then incubated with the secondary antibody conjugated to horseradish peroxidase. After additional washing, signal was detected by chemiluminescence using the ECL kit (Amersham, Pharmacia Biotech) according to the manufacturer's instructions.

Analysis of starch, soluble sugars, free amino acids and proline

Plant samples were homogenised by mortar and pestle, extracted three times with 80% ethanol at 60 °C and centrifuged (14,000×*g*, 15 min). The combined supernatants were stored at −20 °C. Starch content was assessed spectrophotometrically from the insoluble residue following ethanol extraction (Rolletschek et al., 2002). Amino acid samples were analysed by HPLC with fluorescence detection (Rolletschek et al., 2002). Soluble sugars were measured using ion chromatography with pulsed amperometric detection (Rolletschek et al., 2002). Ethanolic seed extracts were used for the mass spectrometric detection of proline, using an HPLC system (pump P680, autosampler ASI-100; Dionex, USA) coupled to a mass spectrometer (LC-1200, Varian, USA). Samples (5 μl/injection) were separated using the Synergy fusion-RP80 reversed phase column (4 μm, 150×1 mm) with methanol (eluent A) and 3% (v/v) formic acid (eluent B). Isocratic separation was effected with 90% A and 10% B for 5 min at a flow rate of 0.1 ml/min. The effluent was split equally, and directed into the electrospray chamber. MS/MS analysis was performed using the following parameters: ESI N2 pressure 53 psi, needle 5800 V, shield 400 V, drying gas 225 °C and 18 psi, detector voltage 1500 V, mass peak with 0.7 amu, scan time 1.5, collision-induced dissociation energy −9 V, collision gas (Ar) pressure 1.5 mTorr, positive ion mode scanning the transition of 116>70. The validity of the method was checked against known standards (Sigma, Germany).

Anthocyanin measurements of mature seeds

Phenolic constituents, including anthocyanins, were analysed on an HPLC system (Alliance 2690, Waters, Eschborn, Germany) combined with a photodiode array detector (FP-920, JASCO, Groβ-Umstadt, Germany). HPLC methods and the extraction of methanol-soluble phenols were as described elsewhere (Mock et al., 1999). Anthocyanin profiles were obtained by extracting chromatograms at 535 nm from the photodiode-array data.

Analysis of sinapate esters

Seed extracts were prepared from 100 mg of dry seed. The tissue was suspended in 80% v/v methanol and disrupted by vigorous shaking in a Bead Beater (Biospec Products, Bartlesville, OK, USA) in the presence of zirconia beads (1 mm diameter). Homogenates were centrifuged and aliquots of the supernatant analysed by HPLC on a 5 μm Nucleosil C18 column (250 mm×4 mm i.d.; Macherey-Nagel, Düren, Germany). A 20 min linear gradient was applied with a flow rate of 1 ml/min from 10 to 50% solvent B (acetonitrile) in solvent A (1.5% *o*-phosphoric acid in water). The compounds were photometrically detected at 330 nm and quantified by external standardisation with authenticated compounds (Milkowski et al., 2004).

Analysis of fatty acids

The fatty acid profile was determined in triplicate by GLC using 5 mg of dry seed per run. Seeds were homogenised in a Retsch mill (Retsch, Hahn) in 1 ml methyl tertiary butyl ether containing 500 μg/ml C17-TAG as internal standard. The lipid extract was transferred to a clean tube and the fatty acids derivatised to the corresponding fatty acid methyl esters (FAMEs) by the addition of trimethyl sulfonium hydroxide (TMSH). The FAMEs were analysed by GLC-FID (HP6890, Agilent Technologies) on capillary columns (Chrompack, WCOT Fused Silica, CP-Wax-52 CB, 25 m, 0.32 mm) using hydrogen as carrier gas. Quantification of the FAMEs was effected by comparison with the peak area of the internal standard.

Heterologous expression of recombinant proteins

The cloning of the cDNA (encoding the full length and the truncated gene product) into expression vector pET23a and the purification of the FUS3 protein has been described previously (Mönke et al., 2004). The FUS3neo coding sequence was amplified using the primers 5′gaggtgaattcgttgatgaaaaagtggaa and 5′gaacctcgagactatataagctatgctttg, and cloned between the pET23a EcoRI and XhoI sites. Protein was synthesised in *Escherichia coli* strain BL21 (DE3), grown at 32 °C after induction with 0.1 mM IPTG for 4 h. Protein purification followed the protocol given by Mönke et al. (2004).

DNA binding assay

Binding of the proteins to biotinylated DNA-oligonucleotides was performed with an ELISA-based method (Mönke et al., 2004). ELISA plates (Falcon) were coated overnight at 4 °C with 5–20 ng/μl of the recombinant proteins diluted in TBS (20 mM Tris–HCl, 150 mM NaCl pH7.5). After three washes with TBS containing 0.1% Tween 20 (TBST), the wells were blocked with 3% BSA dissolved in TBST at room temperature for 2 h. Biotinylated oligonucleotides (0.1 pmol/μl TBS) were added and the plates were incubated at room temperature for 1 h. After three washes, an antibody against biotin conjugated with alkaline phosphatase (Novagen) was added and incubated for 1 h. After three washes, the substrate of the alkaline phosphatase *p*-nitrophenylphosphate dissolved in diethanolamine buffer (pH9.8) was added and the plates were incubated at 37 °C. Enzyme activity was measured at 405 nm with a plate reader (TECAN). Determination of the relative amount of bound protein per well was achieved by ELISA, using a T7 antibody conjugated with alkaline phosphatase. All proteins contain a T7 tag.

Results

EMS mutants suggest the presence of a mutation hot spot in FUS3

The sequencing of several *fus3* EMS mutations has revealed frequent independent point mutations at the exon3/intron3 junction (position 708). Four independent mutants, involving two ecotypes and generated by three independent laboratories, all share the same G to A transition (Fig. 1). The *fus3-1* mutant was identified in the 'Dijon' (Di-G) ecotype in

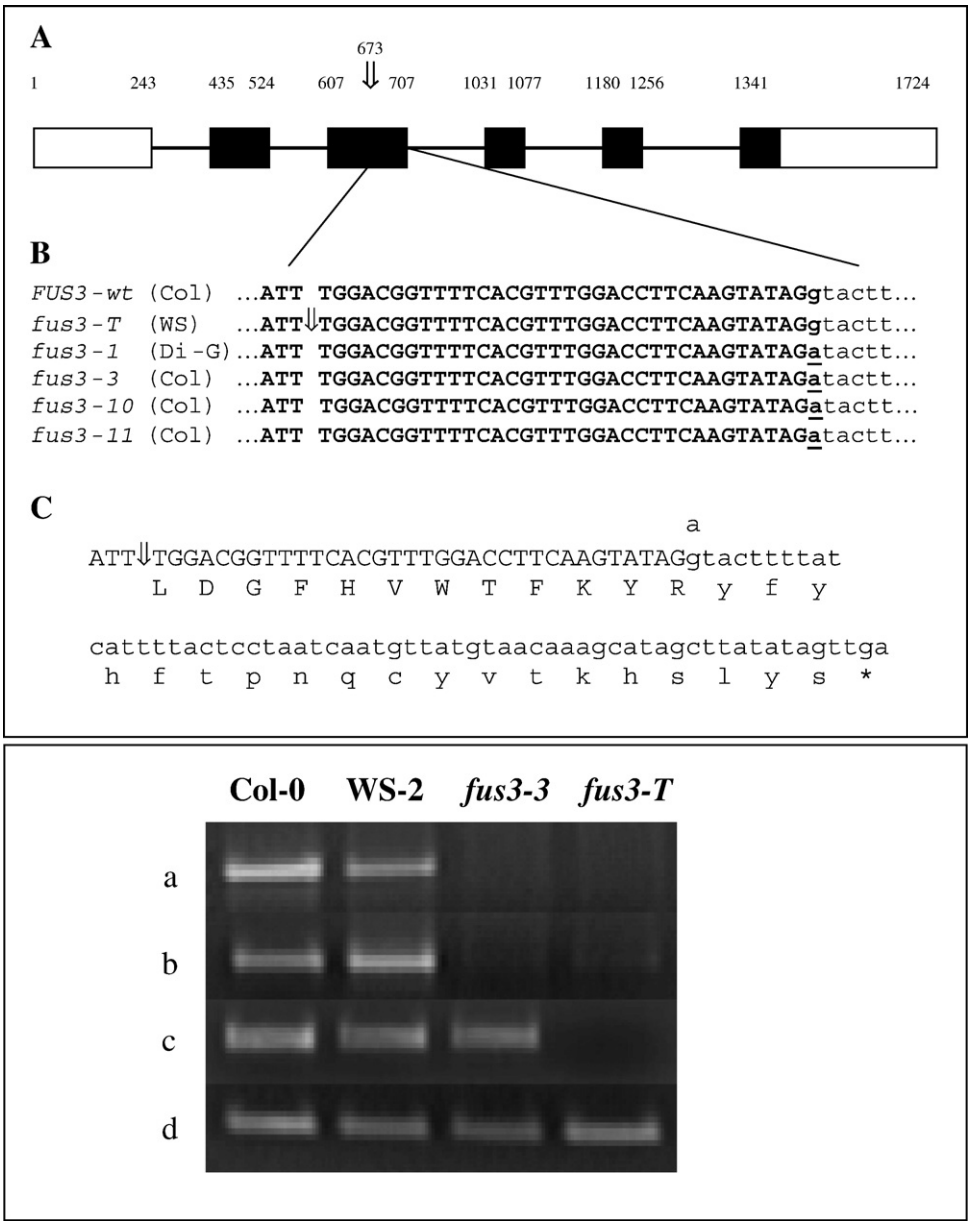
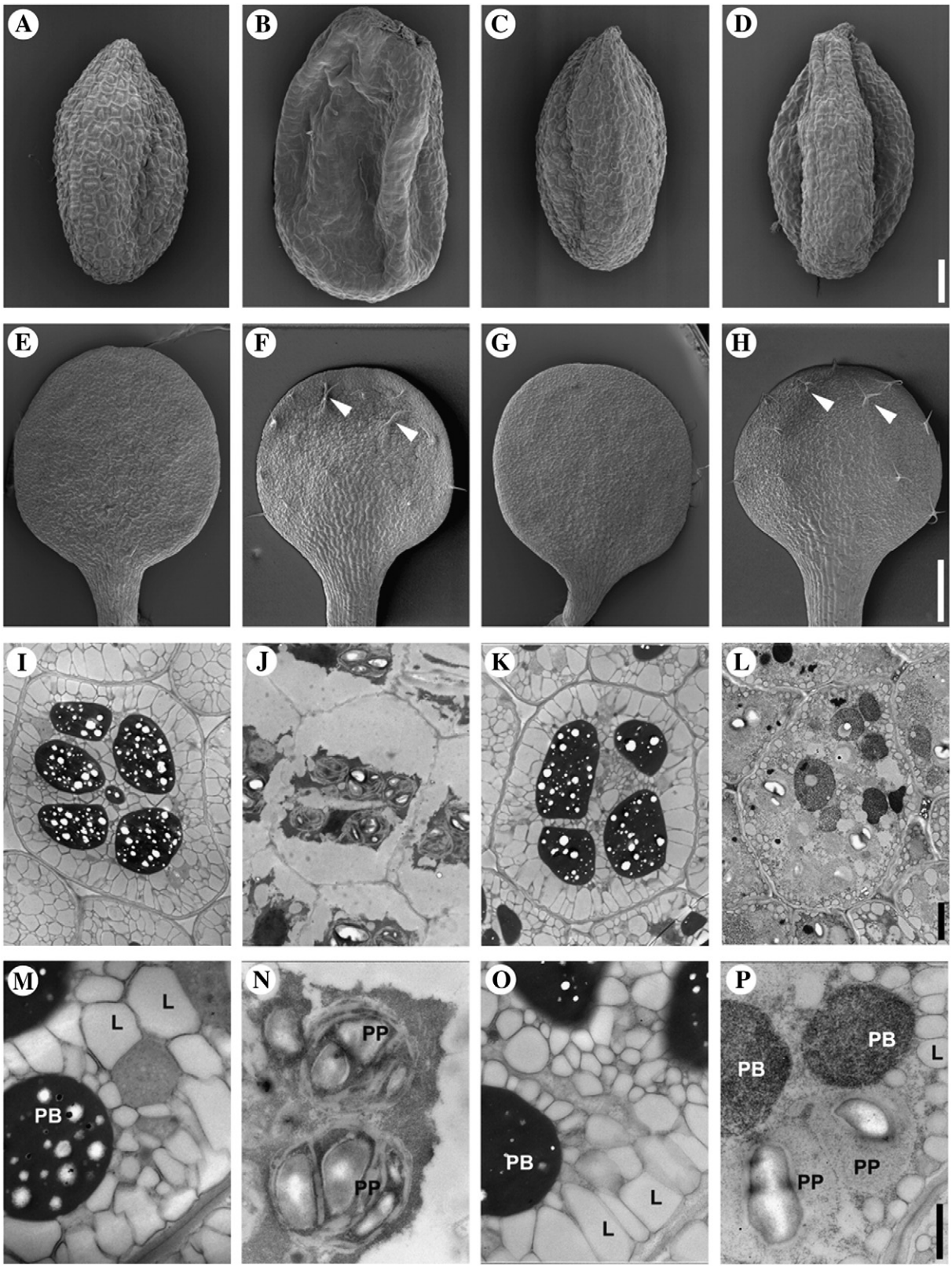


Fig. 1. *Upper panel:* Mutant alleles at *At FUS3*. (A) The exon–intron structure, indicating the exon border positions. The B3 domain regions are shown in *black*. The site of the T-DNA insertion in *fus3-T* at position 673 is *arrowed.* (B) Nucleotide variation around the exon3/intron3 junction. (C) Structure of the C-terminal region of the read-through translation product. *Lower panel:* Transcript detection by RT-PCR in wild types Col-0 and Ws2 *versus* the mutants *fus3-3* and *fus3-T* using the following primers (for primer positions also see Fig. S1): (a) FUS3-Aa and FUS3-KOb, (b) FUS3-Tr and FUS3-KOb, (c) FUS3-Aa and FUS3-Tl, (d) elongation factor EF1Bα was used for loading control.

Fig. 2. Morphological and ultra-structural changes in *fus3-3* and *fus3-T* mutants. (A–H) Scanning electron microscopy of seeds and cotyledons. Wild type Col-0 (A, E) and WS-2 (C, G) produce well-filled ellipsoidal seeds (A, C) and trichome-less cotyledons (E, G). The *fus3-3* mutant produces severely flattened seeds with a severely distorted seed coat structure (B) and cotyledons with trichomes (F). Seeds of *fus3-T* (D) show signs of shrinkage but largely resemble those of wild type. The cotyledons of *fus3-T* carry trichomes (H). (I–P) The ultra-structure of storage parenchyma cells, as revealed by transmission electron microscopy. In wild type Col-0 (I, M) and WS-2 (K, O), parenchymal cells contain large, darkly stained protein bodies and a reticulate network of endomembranes surrounding lipid droplets (M, O). Typical *fus3-3* parenchymal cells have a central clump of collapsed cytoplasm (J, N). Protein bodies, endomembrane network and lipid droplets are absent — instead the cells contain numerous proplastids with starch granules (N). The *fus3-T* parenchymal cells are filled with dense cytoplasm (L). Protein bodies are present but are smaller and stain less intensely than those of the wild type. Lipid droplets are smaller and located only at the cell periphery (compare O and P). Note the presence of proplastids, often containing starch granules (P). Abbreviations: L = lipid droplet, PB = protein body, PP = proplastid. Bars: 50 μm (A–D), 500 μm (E–H), 2 μm (I–L), 1 μm (M–P).

Gatersleben, *fus3-3* in 'Columbia' (Col-0) was isolated in Toronto (Keith et al., 1994), while *fus3-10* and *fus3-11* were both described recently by Raz et al. (2001). Thus seed phenotype-based selection appears to favour the detection of mutations at the splice point consensus. The current analysis focuses on a comparison between *fus3-3* in a Col-0 background and the T-DNA insertion mutant *fus3-T*, in a 'Wassilewskaya' (WS-2) background.



Both mutants exhibit the conventional phenotype

Mature seeds of both mutant lines appear shrunken after desiccation (Figs. 2A–D), although this effect is more pronounced in *fus3-3* than in *fus-3T.* While *fus3-3* seeds are non-viable by this stage, those of *fus3-T* still retain a germination rate of about 70% (Fig. 3A). This figure decreased slowly with prolonged storage at room temperature. When aging was accelerated by storage at 37 °C, the germination rate fell to zero within 31 days, while wild type germination under these conditions of storage was not significantly compromised. The seed coat morphology of the *fus3-T* mutant closely resembles that of the WS-2 wild type, whereas differentiation of the seed coat in the *fus3-3* mutant is heavily distorted (Figs. 2A–D). In both mutants, the cotyledons bear trichomes, a trait which is usually taken as an indicator of the heterochronic or homeotic leafy character (Figs. 2E–H).

Seed dormancy is lost in both mutants

Immature embryos of wild type WS-2 and Col-0 retain some level of dormancy, as their germination rate was less than 20% by 14 days after rescue. In contrast, in both mutant lines, the rate reached ca. 80% by this time (Fig. 3B). While most of the precociously germinated seeds of the mutants went on to develop into viable green seedlings, only very rarely (<0.1%, data not shown) were the equivalent wild type seedlings viable. Wild type seeds typically reached the stage of seed coat rupture (germination criteria according to Bewley and Black, 1994), but did not develop any further. A tendency to vivipary was observed in *fus3-3* supporting the

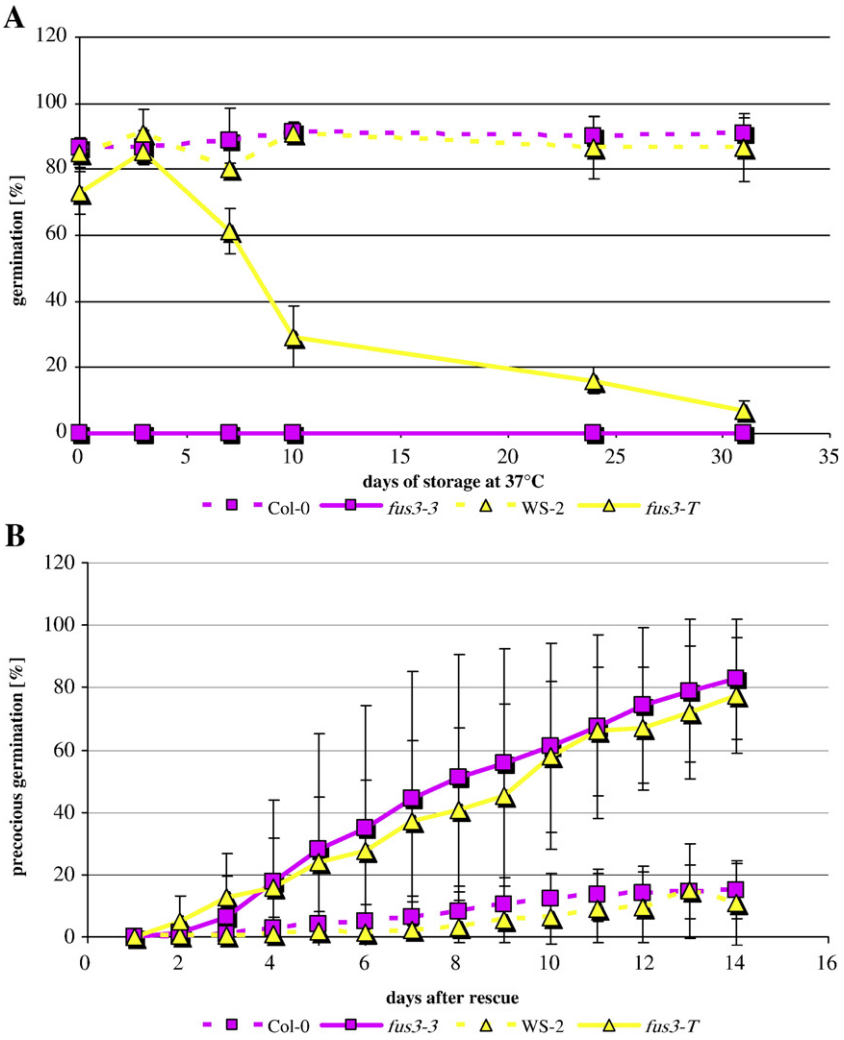


Fig. 3. Drought susceptibility and precocious germination of *FUS3* mutants. (A) Mature seeds of *fus3-3* do not germinate, but *fus3-T* seeds retain a germination rate of >70% immediately after harvest. After storage at 37 °C, the germination rate of *fus3-T* declines, but wild type seeds are largely unaffected by artificial aging. (B) Both mutants show high rates of precocious germination when harvested prematurely (e.g., the early cotyledon stage) and incubated on MS plates. Wild type embryos germinate at a much lower rate. While both mutants develop into seedlings by 17 days after rescue, the vast majority of wild type individuals does not develop much beyond early germination.

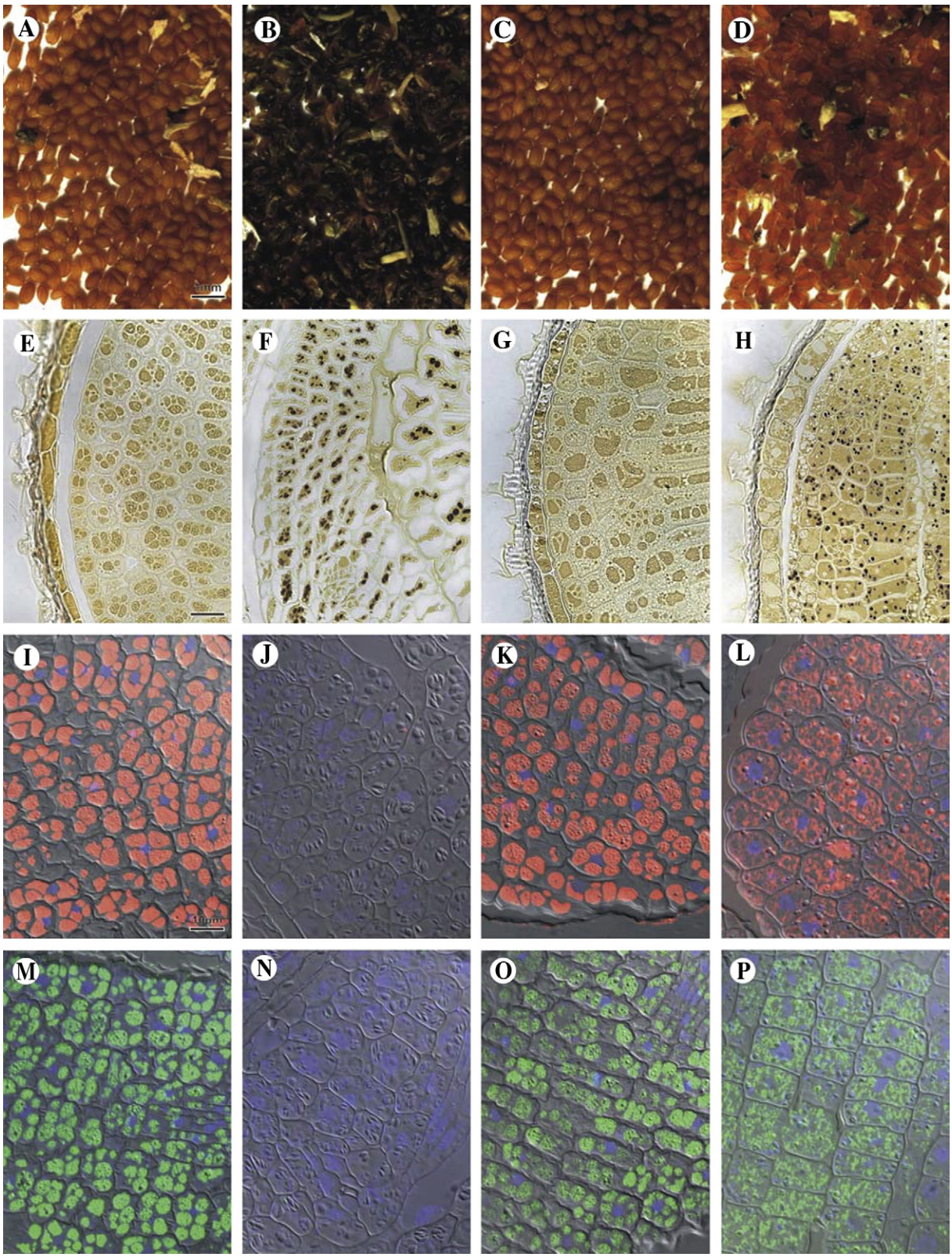


Fig. 4. Morphological and histochemical characterisation of *fus3-3* and *fus3-T* mutants. (A–D) Anthocyanin accumulation in wild type and mutant seeds. Seeds of the *fus3-3* mutant (B) are pigmented due to the presence of anthocyanin. The seeds of *fus3-T* (D) are similar in colouration to the wild type (C). (E–H) Starch accumulation in seeds. The wild type Col-0 (E) and WS-2 (G) contain no starch grains within their cotyledonary cells, but both *fus3-3* (F) and *fus3-T* (H) seeds do deposit starch in their cotyledons. A striking difference between the mutants is the high degree of structural distortion in *fus3-3* (F), while *fus3-T* cells (H) appear morphologically intact. (I–P) Immuno-histochemical staining of cruciferin (I–L, Alexa568) and napin (M–P, Alexa488). While in *fus3-3* (J, N) no storage proteins are detectable, in *fus3-T* both storage proteins are detectable (L, P). Compared to wild type WS-2 (K, O), the protein storage vacuoles in *fus3-T* are abnormal. Napin storage is shown for WS-2 (O) and *fus3-T* (P) in radicle tissue to illustrate the normal distribution of storage proteins throughout the seed organs.

heterochronic character of the mutant, but not in either the wild type or in *fus3-T.*

Storage proteins are present in the mature seeds of the fus3-T mutant

A Western blot analysis demonstrated that, relative to the wild type, the cruciferin content of the mature seed of *fus3-T* was reduced, and that of *fus3-3* contained no detectable cruciferin at all (Fig. S2). Seed section immuno-labelling of cruciferin (Figs. 4I–L) and napin (Figs. 4M–P) confirmed this result. Typical wild type storage parenchyma cells featured several heavily staining large protein bodies, indicative of a high protein content (Figs. 2I–P). The remainder of the cell contained a reticulate network of endomembranes enveloping lipid droplets. Significant alterations in the wild type subcellular structure were most prominent in *fus3-3*. Typically, protein bodies and lipid droplets were completely absent in the storage parenchymal cells of mid to late cotyledon stage, and the cytoplasm was aggregated in the centre of the cell (Figs. 2I–P). Numerous proplastids were present within the cytoplasm, accumulating high amounts of starch (Figs. 4E–H) (as shown also by starch quantification results, Fig. 5B). The *fus3-T* mutant also accumulated starch, but in this case, the starch grains were confined to amyloplasts, since no remnant chloroplast features were detectable (Figs. 2I–P). The storage parenchymal cells of *fus3-T* were filled with dense cytoplasm. Protein bodies present were small and stained less intensely than did those of the wild type, indicative of a lesser protein content. Lipid droplets were also smaller and restricted to the periphery of the cell.

Fus3-T seeds do not accumulate anthocyanin

While mature *fus3-3* seeds become darkened, following the accumulation of anthocyanin, the colour of mature *fus3-T* seeds remains similar to that of the wild type (Figs. 4A–D). No anthocyanin was detectable in the seed of either wild type ecotype. The seed anthocyanin content of *fus3-3* seed was high, while that of *fus3-T* seed was very low, but nevertheless detectable (Fig. 5A).

Both mutants display elevated levels of free amino acids

The seed of both mutants carried an elevated level of free amino acids (Fig. 5B), an effect which was more pronounced for *fus3-3* than for *fus3-T.* The free amino acid composition of *fus3-T* was similar to that in either wild type, whereas for *fus3-3* there was a distinct increase in the concentration of proline (Fig. S4), a response which has been associated with the reaction to a number of environmental stresses (Delauney and Verma, 1993).

Sugar composition and starch content are altered in the mutants

An increased amount of sugars, particularly sucrose (Fig. S3), was present in both mutants, especially *fus3-3* (Fig. 5B). Glucose content was strongly *(fus3-3)* or moderately *(fus3-T)*

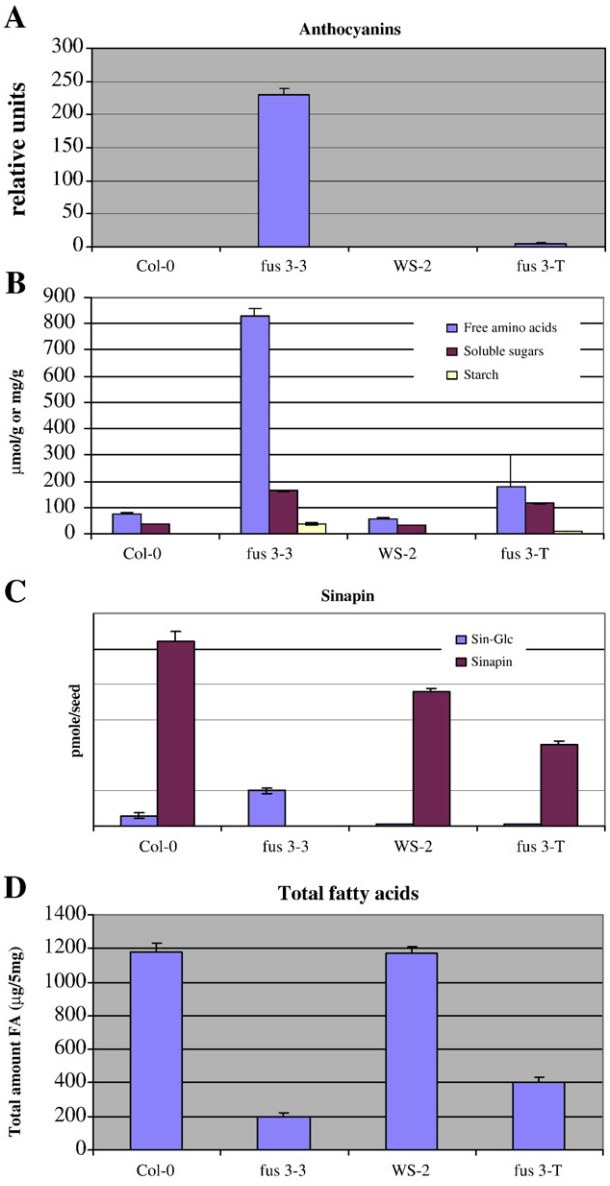


Fig. 5. Metabolite analysis in *fus3-3* and *fus3-T* mutants. (A) Anthocyanin content (relative units) in both wild type (Col-0 and WS-2) and mutants *(fus3-3* and *fus3-T)*. (B) Free amino acids and soluble sugar (µmol/g), and starch (mg/g) content in both wild type and mutants. (C) Sinapin and its precursor sinapoylglucose content (pmol/seed) and in both wild type and mutants. (D) Total fatty acid content (µg/5 mg) in both wild type and mutants. Means calculated from at least three independent measurements. Standard deviations are shown.

increased, while fructose concentration was increased in *fus3-3*, but not in *fus3-T.* Both raffinose and stachyose content was reduced in both mutants (Fig. S3). Some starch was deposited in the seed of both mutants, whereas in the wild type seed no starch was detectable. Again the effect was more pronounced in *fus3-3*. Histological analysis of seed sections confirmed the biochemical measurements (Figs. 4F–I).

Altered sinapate composition

Sinapate ester synthesis is restricted to developing seeds and roughly parallels the timing of storage compound synthesis

(Milkowski et al., 2004). In both mutants the sinapine content of the mature seeds was decreased relative to wild type (Fig. 5C). In *fus3-3*, the level fell below the detection limit. The radical decrease in the sinapine content of *fus3-3* seed was accompanied by a significant increase in the level of 1-*O*-sinapoylglucose, the precursor of sinapine. Hence, the biochemical phenotype of *fus3-3* is reminiscent of that of the *sng2* mutant, in which sinapoylglucose, rather than sinapine, accumulates, as a result of a lesion in *SNG2* (*At5g09640*) which encodes sinapoylglucose:choline sinapoyltransferase (SCT; EC 2.3.1.91) (Shirley et al., 2001). RT-PCR transcript analysis also identified a significant decrease in the presence of the seed-specific SCT message in the *fus3-3* mutant, and a less pronounced decrease in *fus3-T* (Fig. S6). Levels of UDP glucose:sinapate glucosyltransferase (SGT, EC 2.4.1.120, *At3g21560*) were unaffected by either mutation (Fig. S6).

The changes in fatty acid composition are concentrated on long chain species

The total fatty acid content of both mutants is both quantitatively and qualitatively different from that of the wild type. The level was substantially reduced in the *fus3-3*, and less so in the *fus3-T* mutant (Fig. 5D). The mutants did not differ from the wild type with respect to either palmitic (16:0) or stearic (18:0) acid content, but the level of oleic acid (18:1) was moderately increased and those of linoleic (18:2) and α-linolenic (18:3) acid reduced. The most pronounced change was in the content of eicosenoic acid (20:1), the major seed storage species (Finkelstein and Somerville, 1990), which was severely reduced in *fus3-3*, but less so in *fus3-T* seeds (Fig. S5).

Fus3-3 is a gain of function mutant

The lack of an amplifiable *FUS3* transcript in *fus3-T* silique RNA suggested that this allele is a null mutant (Fig. 1). In contrast, the derived product described by Luerssen et al. (1998), in which in-frame sequences of intron 3 are recruited in the presence of the mutated splice point at the end of exon 3, was present in the silique RNA of *fus3-3*. The read-through translation product is terminated after 19 additional codons (Fig. 1). The aberrant gene product was heterologously expressed in *E. coli* and the isolated protein used for DNA binding studies with both intact and compromised RY target motifs (Fig. 6). The outcome of these experiments is that the neomorphic protein still recognises the RY motif, but exhibits a lower affinity and a reduced specificity compared to the wild type FUS3 gene product. The F1 hybrids *fus3-3*×*fus-3T* all showed the *fus3-3* phenotype (data not shown), demonstrating that the *fus3-3* neomorphic protein has a phenotypic effect on the loss of function *fus3-T* mutant.

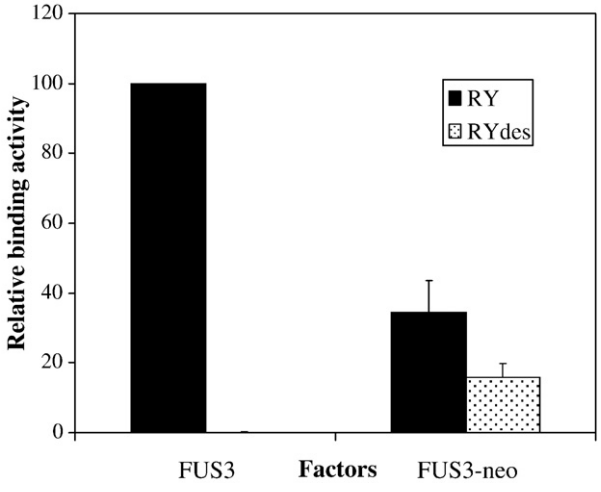


Fig. 6. ELISA-based *in vitro* binding studies. Intact FUS3 and the truncated neomorphic protein (FUS3-neo) were tested for their ability to interact with biotinylated DNA-oligonucleotides containing either the RY wild type motif (CATGCATG) or the compromised motif (TGACCAGC).

Table 1
Dissection of a complex seed phenotype: phenotypic characteristics of the *fus3-3* and *fus3-T* mutants, highlighting the intermediate nature of *fus3-T*

Wild type	*fus3-T*	*fus3-3*
Intact transcription factor	Loss of function mutant	Aberrantly truncated neomorph
Seed differentiation		Vegetative differentiation
Viable seeds		Lethal seeds
Seed characteristic composition of amino acids, free sugars, sinapate esters, fatty acids	Intermediate composition of amino acids, free sugars, sinapate esters, fatty acids	Aberrant composition of amino acids, free sugars, sinapate esters, fatty acids
No anthocyanin	Very low anthocyanin content	High anthocyanin content
Normal, non shrunken seeds	Moderately shrunken seeds	Severely shrunken seeds
Seed coat morphologically intact	Seed coat morphologically intact	Distorted seed coat structure
Desiccation tolerant	Susceptible to prolonged seed storage	Desiccation intolerant
No starch	Moderate starch content	High starch content
Normal storage protein content	Reduced storage protein content	No detectable storage proteins
Dormant	Non dormant	
Hairless cotyledons	Hairy leafy cotyledon	

Fus3-T represents an intermediate phenotype

The overall phenotypic observations are summarised in Table 1, which highlights the commonalities and differences between the two mutants. The most consistent interpretation is that the loss of function *fus3-T* mutant generates an intermediate phenotype, in which only a moderate disturbance of most seed specific differentiation processes occurs. In contrast, the splice point mutation in *fus3-3*, which results in the translation of a truncated aberrant gene product, causes a more complex disturbance to the process of seed development due to a severe mis-differentiation.

Discussion

Mutant alleles of *FUS3* represent an important resource for the analysis of transcriptional regulation during seed development. To date, only chemically induced mutants have been available

(Müller, 1963, Müller and Heidecker, 1968; Keith et al., 1994). Since the cloning of *FUS3* (Luerssen et al., 1998), a number of identical mutation sites have been detected in independently selected mutants in both the Di-G and Col-0 background (Keith et al., 1994; Raz et al., 2001). The T-DNA insertion *FUS3* mutant, which was identified without any recourse to phenotyping, was expected to show a similar phenotype to the established EMS mutants, but instead appears to generate an intermediate phenotype. This has led us to undertake a more comprehensive comparison between the splice point allele *fus3-3* and the T-DNA insertion allele *fus3-T*.

Some phenotypic aspects are shared between the two mutants. Both lose embryo derived dormancy and their development of cotyledonary trichomes indicates the heterochronic or homeotic leafy character (Meinke et al., 1994, Keith et al., 1994). However, the characteristic anthocyanin accumulation in *fus3-3* (and other chemically induced mutants) is virtually absent in *fus3-T*. Unlike *fus3-3* seeds, mature *fus3-T* seeds are viable, but still lose viability as they age. Moreover, the cells of maturing *fus3-T* embryos can differentiate into typical storage cells, whereas *fus3-3* cells maintain a leaf-like state of differentiation. Finally, several phenotypic features of *fus3-T* are intermediate between *fus3-3* and wild type, including the shrunken seed phenotype, the accumulation of various storage compounds, and the composition of free amino acids, free sugars and sinapate esters (Table 1).

A possible hypothesis to explain these observations is that the *fus3-T* mutant carries a weak *FUS3* allele. To dispose of this hypothesis, it was necessary to demonstrate that the T-DNA insertion results in a true loss of function. The absence of a functional transcript is taken as proof that no FUS3 translation product can be present. The aberrant transcripts produced by the splice point alleles in the Di-G background have been described elsewhere (Luerssen et al., 1998), and a similar transcript was detectable in *fus3-3*. This encodes a truncated protein carrying 19 additional, intron-encoded residues, and the resulting neomorphic translation product is likely responsible for the differences between the EMS and the intermediate effect T-DNA mutants. This model explains the dominance of the *fus3-3* over the *fus-3T* lesion, but also predicts a *fus3-3* type phenotype for the *fus3-3/+* hybrid, which is not the case in practice. The B3-domain proteins FUS3 and ABI3 regulate the expression of their gene target *via* a specific direct interaction with RY elements (Reidt et al., 2000; Kroj et al., 2003, Mönke et al., 2004), although kinetic analyses indicate that gene activation by FUS3 involves a more complex process (Kagaya et al., 2005a). The neomorphic *fus3-3* protein retains only the N-terminal part of the DNA-binding B3 domain and lacks the putative activation domain previously identified within the 53 C-terminal amino acids of the FUS3 protein (Kagaya et al., 2005a). The loss of the activation domain suggests that the function of the truncated mutant protein does not depend on gene activation, but is more likely due to an aberrant DNA binding. DNA binding experiments have demonstrated that the neomorphic *fus3-3* protein retains some DNA binding capacity most likely mediated by the retained N-terminal part of the DNA binding B3 domain encoded by the second and the third exon. However, its binding capacity is weaker and much less specific than that of the intact FUS3 protein. Thus in the *fus3-3/+* heterozygote, the putative threshold sensitive effects of the neomorphic protein are quite probably masked. Thus we suggest that the complex phenotype of the splice point mutants such as *fus3-3* is in part due to the loss of FUS3 function, and in part to the addition of novel features determined by the presence of the truncated neomorphic protein. In contrast, the T-DNA insertion mutant *fus3-T* represents a true null mutant, showing only the loss of function effects.

An equivalent situation has been described recently by the *At* transcription factor BODENLOS (Hamann et al., 2006), in which a single G to A transition results in a predicted proline to serine change in a highly conserved domain, leading to a gain of function mutation in the auxin response regulator IAA12. The mutant is semidominant during postembryonic development but recessive with respect to embryonic phenotype. It appears that the copy number of the mutant allele is a critical determinant, both in the absence and presence of wild type alleles (Hamann et al., 2006).

The anthocyanin accumulation characteristic of *fusca* mutants is a derived pleiotropic trait, common to splice point alleles encoding truncated and mis-elongated neomorphic proteins. It is likely caused by a failure of the cotyledon cells to differentiate. These cells do not differentiate into a proper storage organ, but remain leaf-like. An aspect of this phenomenon is the presence of vacuoles which are the natural targets for anthocyanins. Anthocyanin synthesis itself may be a consequence of desiccation stress. The differentiation of embryo organs into seed storage organs is a critical step for the seed to acquire desiccation tolerance. This differentiation is retained in the *fus3-T* mutant, which is non-lethal. Thus we deduce that the wild type function of the FUS3 protein cannot be directly involved in the acquisition of seed desiccation tolerance.

On the basis of our observations, the direct effects of the FUS3 gene product must be restricted to one or more of the following: the induction of embryo-dependent dormancy and the inhibition of precocious germination; the transcriptional induction of storage protein and storage lipid synthesis (although it cannot be the sole player in this process) as well as the determination of the cellular differentiation of cotyledonary epidermis cells, preventing trichome formation. These conclusions are at least partly consistent with the previously defined TTG1-mediated function of FUS3. The expression of *FUS3* has mainly been detected in the protodermal tissue of the embryo. The specific expression of *FUS3* in the protodermal cell layer can rescue most of the *fus3* phenotypes, including the accumulation of seed storage proteins in different cell layers of the embryo. This L1-specific expression pattern is however difficult to reconcile with a direct effect of FUS3 on the promoter of seed storage proteins encoding genes throughout the embryo (Reidt et al., 2000; Kroj et al., 2003; Mönke et al., 2004). It has been proposed that FUS3 would act indirectly through the negative regulation of TTG1 and acts as nexus of hormone action during embryogenesis (Gazzarini et al., 2004; Tsuchiya et al., 2004; Curaba et al., 2004). However, a remaining discrepancy is the development of trichomes on the cotyledons of the *fus3-T* mutant, without the characteristic

massive accumulation of anthocyanin, since both these processes are positively regulated by the FUS3 suppressed TTG1 protein (Tsuchiya et al., 2004). When a loss of function *ttg1* mutation is introduced into a *fus3* mutant, a number of *fus3*-related phenotypes are rescued. This indicates that a functional *TTG1* gene is required for the manifestation of the *fus3* mutant phenotype (Tsuchiya et al., 2004). However, this FUS3-TTG1 interaction includes both maternal and zygotic interactions, indicating a rather complex functional relationship between both gene products during seed development. This complexity might partially explain the contradicting observation that both positively TTG1 regulated features as trichome formation on cotyledons and anthocyanin accumulation behave contrary in the *fus3-T* mutant.

Sensu stricto, *fus3-T* is not a *fusca* mutant, as it lacks the anthocyanin accumulation trait. Moreover, the screening procedure at least partially explains the detection of the described mutation at the splice point of the third exon-intron border. The pleiotropic effects caused by the expression of the neomorphic protein forces a re-evaluation of the regulatory networks active during seed development, which have in part been based on the assumption that the *fus3-3* mutant is a loss of function allele (e.g., Bäumlein et al., 1994; Keith et al., 1994; Kroj et al., 2003; To et al., 2005). In summary, the direct effects of FUS3 are restricted to embryo-derived dormancy *via* the negative regulation of the GA-synthesis, and on the determination of cotyledon epidermis cell identity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2008.01.034.

References

Bäumlein, H., Wobus, U., Pustell, H., Kafatos, F.C., 1986. The legumin gene family: structure of a B-type gene of *Vicia faba* and a possible legumin gene specific regulatory element. Nucleic Acids Res. 14, 2707–2720.

Bäumlein, H., Nagy, I., Villarroel, R., Inze, D., Wobus, U., 1992. *Cis*-analysis of a seed promoter: The seed protein gene specific RY repeat CATGCATG is essential for expression of a legumin gene. Plant J. 2, 233–239.

Bäumlein, H., Miserá, S., Luerssen, H., Kölle, K., Horstmann, C., Wobus, U., Müller, A.J., 1994. The *FUS3* gene of *Arabidopsis thaliana* is a regulator of gene expression during late embryogenesis. Plant J. 6, 379–387.

Bewley, J.D., Black, M., 1994. Seeds. Physiology of Development and Germination, 2nd Edition. Plenum Press, New York.

Borisjuk, N., Sitailo, L., Adler, K., Malysheva, L., Tewes, A., Borisjuk, L., Manteuffel, R., 1998. Calreticulin expression in plant cells: developmental regulation, tissue specificity and intracellular distribution. Planta 206, 504–514.

Brocard-Gifford, I.M., Lynch, T.J., Finkelstein, R.R., 2003. Regulatory networks in seeds integrating developmental, abscisic acid, sugar and light signalling. Plant Physiol. 131, 78–92.

Curaba, J., Moritz, T., Blervaque, R., Parcy, F., Raz, V., Herzog, M., Vachon, G., 2004. *AtGA3ox2*, a key gene responsible for bioactive gibberellin biosynthesis, is regulated during embryogenesis by *LEAFY COTYLEDON2* and *FUSCA3* in *Arabidopsis*. Plant Physiol. 136, 3660–3669.

Delauney, A.J., Verma, D.P.S., 1993. Proline biosynthesis and osmoregulation in plants. Plant J. 4, 215–223.

Dickinson, C.D., Evans, R.P., Nielsen, N.C., 1988. RY repeats are conserved in the 5′-flanking regions of legume seed-protein genes. Nucleic Acids Res. 16, 371.

Finkelstein, R.R., Somerville, C.R., 1990. Three classes of abscisic acid (ABA)-insensitive mutations of *Arabidopsis* define genes that control overlapping subsets of ABA responses. Plant Physiol. 94, 1172–1179.

Gazzarini, S., Tsuchiya, Y., Lumba, S., Okamoto, M., McCourt, P., 2004. The transcription factor FUSCA3 controls developmental timing in *Arabidopsis* through the hormones gibberellin and abscisic acid. Dev. Cell 7, 373–385.

Giraudat, J., Hauge, B.M., Valon, C., Smalle, J., Parcy, F., Goodman, H.M., 1992. Isolation of the *Arabidopsis ABI3* gene by positional cloning. Plant Cell 4, 1251–1261.

Hamann, T., Benkova, E., Bäuerle, I., Kientz, M., Jürgens, G., 2006. The *Arabidopsis BODENLOS* gene encodes an auxin response protein inhibiting MONOPTEROS-mediated embryo patterning. Genes Dev. 16, 1610–1615.

Kagaya, Y., Okuda, R., Ban, A., Toyoshima, R., Tsutsumida, K., Usui, H., Yamamoto, A., Hattori, T., 2005a. Indirect ABA-dependent regulation of seed storage protein genes by FUSCA3 transcription factor in *Arabidopsis*. Plant Cell Physiol. 46, 300–311.

Kagaya, Y., Toyoshima, R., Okuda, R., Usui, H., Yamamoto, A., Hattori, T., 2005b. LEAFY COTYLEDON1 controls seed storage protein genes through its regulation of FUSCA3 and ABSCISIC ACID INSENSITIVE3. Plant Cell Physiol. 46, 399–406.

Keith, K., Kraml, M., Dengler, N.G., McCourt, P., 1994. *fusca3*: a heterochronic mutation affecting late embryo development in *Arabidopsis*. Plant Cell 6, 589–600.

Koornneef, M., Karssen, C.M., 1994. Seed dormancy and germination. In: Meyerowitz, E.M., Somerville, C.R. (Eds.), *Arabidopsis*. Cold Spring Harbor Press, Cold Spring Harbor, NY.

Koornneef, M., Hanhart, C.J., Hilhorst, H.W.M., Karssen, C.M., 1989. *In vivo* inhibition of seed development and reserve accumulation in recombinants of abscisic acid biosynthesis and responsiveness mutants in *Arabidopsis thaliana*. Plant Physiol. 90, 463–469.

Kotak, S., Vierling, E., Bäumlein, H., von Koskull-Döring, P., 2007. A novel transcriptional cascade regulating expression of heat stress proteins during seed development of *Arabidopsis*. Plant Cell 19, 182–195.

Kroj, T., Savino, G., Valon, C., Giraudat, J., Parcy, F., 2003. Regulation of storage protein gene expression in *Arabidopsis*. Development 130, 6065–6073.

Laemmli, M.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Luerssen, H., Kirik, V., Hermann, P., Miséra, S., 1998. The *FUSCA3* gene of *Arabidopsis thaliana* encodes a product with partial homology to VP1/ABI3-like regulatory proteins. Plant J. 15, 755–764.

McCarty, D.R., Hattori, T., Carson, C.B., Vasil, V., Lazar, M., Vasil, I.K., 1991. The *viviparous*-1 developmental gene of maize encodes a novel transcription activator. Cell 66, 895–905.

Meinke, D.W., 1992. A homeotic mutant of *Arabidopsis thaliana* with leafy cotyledons. Science 258, 1647–1650.

Meinke, D.W., Franzmann, L.H., Nickle, T.C., Yeung, E.C., 1994. *Leafy* Cotyledon mutants of *Arabidopsis*. Plant Cell 6, 1049–1064.

Milkowski, C., Baumert, A., Schmidt, D., Nehlin, L., Strack, D., 2004. Molecular regulation of sinapate ester metabolism in *Brassica napus*: expression of genes, properties of the encoded proteins and correlation of enzyme activities with metabolite accumulation. Plant J. 38, 80–92.

Misera, S., Müller, A., Weiland-Heidecker, U., Jürgens, G., 1994. The *FUSCA* genes of *Arabidopsis*: negative regulators of light responses. Mol. Gen. Genet. 244, 242–252.

Mock, H.P., Heller, W., Molina, A., Neubohn, B., Sandermann, H., Grimm, B., 1999. Expression of uroporphyrinogen decarboxylase or coproporphyrinogen oxidase antisense RNA in tobacco induces pathogen defense responses conferring increased resistance to TMV. J. Biol. Chem. 27, 4231–4238.

Mönke, G., Altschmied, L., Tewes, A., Reidt, W., Mock, H.P., Bäumlein, H., Conrad, U., 2004. Seed-specific transcription factors ABI3 and FUS3: molecular interaction with DNA. Planta 219, 158–166.

Müller, A.J., 1963. Embryonentest zum Nachweis rezessiver Lethalfaktoren bei *Arabidopsis thaliana*. Biologisches Zentralblatt 82, 133–163.

Müller, A.J., Heidecker, U., 1968. Lebensfähige und letale *fusca* Mutanten bei *Arabidopsis thaliana*. Arabidopsis Informations Service 5, 54–55.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. J. Plant Physiol. 15, 473–497.

Parcy, F., Valon, C., Raynal, M., Gaubier-Comella, A., Delsney, M., Giraudat, J., 1994. Regulation of gene expression programs during seed development: roles of ABI3 locus and of endogenous abscisic acid. Plant Cell 6, 1567–1582.

Parcy, F., Valon, C., Kohara, A., Miserá, A., Giraudat, J., 1997. The *ABSCISIC ACID2*, *FUSCA3* and *LEAFY COTYLEDON1* genes act in concert to control multiple aspects of *Arabidopsis* seed development. Plant Cell 9, 1265–1277.

Paz-Arez, et al., 2002. REGIA, an EU project on functional genomics of transcription factors from *Arabidopsis thaliana*. Comparative and Functional Genomics 3, 102–108.

Raz, V., Bergervoet, J.H., Koornneef, M., 2001. Sequential steps for developmental arrest in *Arabidopsis* seeds. Development 128, 243–252.

Reidt, W., Wohlfarth, T., Ellerström, M., Czihal, A., Tewes, A., Ezcurra, I., Rask, L., Bäumlein, H., 2000. Gene regulation during late embryogenesis: the RY motif of maturation-specific gene promoters is a direct target of the FUS3 gene product. Plant J. 21, 401–408.

Reidt, W., Ellerström, M., Kölle, K., Tewes, A., Tiedemann, J., Altschmied, L., Bäumlein, H., 2001. FUS3-dependent gene regulation during late embryogenesis. J. Plant Physiol. 158, 411–418.

Rider, S.D., Henderson, J.T., Jerome, R.E., Edenberg, H.J., Romero-Severson, R., Ogas, J., 2003. Coordinate repression of regulators of embryonic identity by PICKLE during germination in *Arabidopsis*. Plant J. 35, 33–43.

Rolletschek, H., Hajirezaei, M., Wobus, U., Weber, H., 2002. Antisense-inhibition of ADP-glucose pyrophosphorylase in *Vicia narbonensis* seeds increases soluble sugars and lead to higher water and nitrogen uptake. Planta 214, 954–964.

Santos Mendoza, M., Dubreucq, B., Miquel, M., Caboche, M., Lepiniec, L., 2005. *LEAFY COTYLEDON2* activation is sufficient to trigger the accumulation of oil and seed specific mRNAs in *Arabidopsis* leaves. FEBS Lett. 579, 3550–3560.

Shirley, A.M., McMichael, C.M., Chapple, C., 2001. The *sng2* mutant of *Arabidopsis* is defective in the gene encoding the serine carboxypeptidase-like protein sinapoylglucose: choline sinapoyl-transferase. Plant J. 28, 83–94.

Stone, S.L., Kwong, L.W., Yee, K.M., Pelletier, J., Lepiniec, L., Fischer, R.L., Goldberg, R.B., Harada, J.J., 2001. *LEAFY COTYLEDON2* encodes a B3 domain transcription factor that induces embryo development. Proc. Natl. Acad. Sci. U. S. A. 98, 11806–11811.

Sussman, M.R., Amasino, R.M., Young, J.C., Krysan, P.J., Austin-Phillips, S., 2000. The *Arabidopsis* knockout facility at the University of Wisconsin–Madison. Plant Physiol. 124, 1465–1467.

Tiedemann, J., Neubohn, B., Müntz, K., 2000. Different functions of vicilin and legumin are reflected in the histopattern of globulin mobilization during germination of vetch (*Vicia sativa* L.). Planta 211, 1–12.

To, A., Valon, C., Savino, G., Guilleminot, J., Devic, M., Giraudat, J., Parcy, F., 2005. A network of local and redundant gene regulation governs *Arabidopsis* seed maturation. Plant Cell 18, 1642–1651.

Tsuchiya, Y., Nambara, E., Naito, S., McCourt, P., 2004. The FUS3 transcription factor functions through the epidermal regulator TTG1 during embryogenesis in *Arabidopsis*. Plant J. 37, 73–81.

Wang, H., Guo, J., Lambert, K.N., Lin, Y., 2007. Developmental control of *Arabidopsis* seed oil biosynthesis. Planta 226, 773–783.

West, M.A.L., Yee, K.M., Danao, J., Zimmerman, J.L., Fischer, R.L., Goldberg, R.B., Harada, J.J., 1994. *LEAFY COTYLEDON1* is an essential regulator of late embryogenesis and cotyledon identity in *Arabidopsis*. Plant Cell 6, 1731–1745.